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PROVISIONAL SPECIFICATION

Applicant(s):

SYDNEY IVF PTY LTD

Invention Title:

FEMALE GERM-LINE-CELL MITOCHONDRIAL GENOME ANALYSIS I

The invention is described in the following statement:

FEMALE GERM-LINE-CELL MITOCHONDRIAL GENOME ANALYSIS I

TECHNICAL FIELD

The present invention relates to a method of isolating a cytoplasmic fraction from an oocyte which does not impair the capacity of the oocyte to be fertilized.

The invention also relates to methods for analysing the mitochondrial genome of mitochondria in an oocyte, for detecting nucleotide sequence mutation in the mitochondrial genome of an oocyte, for predicting whether progeny descended from a fertilized oocyte will suffer a disease or dysfunction which is caused by, or associated with, a mutation in the mitochondrial genome of the oocyte, and for screening, or for selecting, an oocyte for fertilization.

The invention also relates to methods for analysing the mitochondrial genome of mitochondria in an embryonic cell, for detecting nucleotide sequence mutation in the mitochondrial genome of an embryonic cell, for predicting whether progeny descended from an embryonic cell will suffer a disease or dysfunction which is caused by, or associated with, a mutation in the mitochondrial genome of the embryonic cell, and for selecting an embryo for embryo transfer.

The invention also relates to a kit including at least one oligonucleotide primer for use in the methods of the invention.

BACKGROUND ART

The known methods for the analysis of the oocyte cytoplasm have necessitated the destruction of the oocyte (1-3). Consequently, although the information retrieved in the analysis may be of particular interest, the investigator is unable to further study the oocyte as a complete cellular unit in the context of the information

retrieved from the analysis.

Methods for introducing extraneous materials into eukaryotic cells, including for example, micro-injection, are known in the art (4). These methods are based on the use of micro-manipulator apparatus which are designed for the specific purpose of introducing extraneous materials into eukaroytic cells, and which typically comprise a vacuum means for securing a single oocyte and an injection pipette for introducing the extraneous material into the secured oocyte.

DESCRIPTION OF THE INVENTION

The present inventors recognised that to provide information in relation to a cytoplasmic fraction of an oocyte, in particular, oocyte mitochondria, and yet maintain the oocyte as a viable cellular unit, is a significant hurdle to the study of cytoplasmic mechanisms in the oocyte, in particular, those mechanisms which are mediated by mitochondria and which are the cause of, or are associated with, dysfunction or disease, either in the oocyte itself, or in progeny which is descended from the fertilized oocyte.

The present inventors recognised that it might be possible to isolate a cytoplasmic fraction from an oocyte without impairing the capacity of the oocyte to be fertilized. Further, the present inventors recognised that it might be possible to retrieve mitochondria from the oocyte in the cytoplasmic fraction, without interfering significantly with the metabolism of the oocyte.

Further the present inventors surprisingly found that prior art methods and apparatus, which are specifically designed for introducing extraneous materials into eukaryotic cells, are of use in retrieving or isolating a cytoplasmic fraction from an oocyte, without impairing the capacity of the oocyte to be fertilized and the capacity to

develop.

Thus in one aspect, the invention relates to a method of isolating a cytoplasmic fraction from an oocyte which does not impair the capacity of the oocyte to be fertilized, the method including the step of releasing a cytoplasmic fraction from the oocyte. Typically, the cytoplasmic fraction is released from the oocyte using a releasing means. Typically the volume of the cytoplasmic fraction drawn into the releasing means is less than 10 pL and preferably 8 pL. Preferably the releasing means includes at least an injection pipette. More preferably, the injection pipette is an intra cytoplasmic sperm injection (ICSI) pipette and preferably the cytoplasmic fraction is drawn approximately 100μm into the pipette.

In a second aspect, the invention relates to a method of isolating a cytoplasmic fraction from an oocyte which does not impair the capacity of the oocyte to be fertilized, the method including the following steps:

a) drawing the fraction from the oocyte into a releasing means; and
b) isolating the fraction from the oocyte.

In accordance with the second aspect of the invention, the volume of the cytoplasmic fraction drawn into the releasing means from the oocyte is typically less than 10 pL and preferably 8 pL. Preferably, the releasing means includes at least an ICSI pipette and preferably the cytoplasmic fraction is drawn approximately 100μm into the pipette.

The drawing of the cytoplasmic fraction from the oocyte into the releasing means in accordance with the second aspect of the invention typically forms an extrusion of cytoplasmic contents between the releasing means and the oocyte. The cytoplasmic fraction is isolated from the oocyte by gently stretching or shearing the extrusion so as to separate the extrusion. Preferably the cytoplasmic

fraction is isolated by stretching the extrusion.

Typically, the cytoplasmic fraction obtained in accordance with the second aspect of the invention contains cytoplasmic organelles, and includes or consists of a sample of the oocyte's mitochondria.

It is known that the mitochondria located in an oocyte are a template from which all mitochondria in the progeny descended from the fertilized oocyte are derived. The present inventors recognised that a method for isolating a cytoplasmic fraction from an oocyte, without impairing the capacity of the oocyte to be fertilized, would allow the skilled worker to study the relationship between the integrity of the mitochondrial genome and the function of both the oocyte, and the progeny descended from the fertilized oocyte.

Thus, in a third aspect, the invention relates to a method of analysing the mitochondrial genome of mitochondria located in an oocyte which does not impair the capacity of the oocyte to be fertilized, the method including the following steps:

a) drawing a cytoplasmic fraction which includes a sample of mitochondria from the oocyte, into a releasing means;
b) isolating the fraction from the oocyte; and
c) analysing the mitochondrial genome of the mitochondria in the fraction.

In accordance with the third aspect of the invention, the mitochondrial genome of the mitochondria which are in the fraction isolated from the oocyte, may be analysed by use of standard techniques known in the art, and exemplified in the accompanying Examples, including techniques, or a combination of techniques, selected from the group of techniques consisting of the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing.

In accordance with the third aspect of the invention,

the volume of the cytoplasmic fraction drawn into the releasing means from the oocyte is typically less than 10 pL and preferably 8 pL. Preferably, the releasing means includes at least an ICSI pipette and preferably the cytoplasmic fraction is drawn approximately 100μm into the pipette.

The drawing of the cytoplasmic fraction from the oocyte into the releasing means in accordance with the third aspect of the inven ion typically forms an extrusion of cytoplasmic contents between the distal end of the releasing means and the oocyte. The cytoplasmic fraction is isolated from the oocyte by gently stretching or shearing the extrusion so as to separate the extrusion. Preferably the cytoplasmic fraction is isolated by stretching the extrusion.

Mutation of the mitochondrial genome is known to cause, or at least to be associated with, dysfunction or disease. For example, the nucleotide sequence deletion at from nucleotide position number 8470 to 13,446 of the mitochondrial genome, the so called "5kb common deletion" (5), is understood to be associated with Kearns-Sayre syndrome (KSS) and chronic progressive external opthalmoplegia (CPEO) (6-9). Other disease causing-deletions, which may or may not be observed with the common deletion include a 7.4 kb deletion and 10.4 kb deletion/insertion in the mitochondrial genome of brain and heart (10-11), as well as various point mutations (12). The deletion has also been observed in human tissue including skeletal muscle, heart, brain, oocytes, leukocytes, retina and ovaries (1, 2, 13-20).

Over 40 pathogenic point mutations of the mitochondrial genome (21) have been associated with a broad spectrum of degenerative diseases involving the central nervous system, heart, muscle, endocrine system, kidney and liver (22). Diseases associated with point mutations include Leigh Syndrome, MELAS (mitochonrdial

encephalomyopathy, lactic acidosis and stroke like episodes), MERRF (myoclonus epilepsy with ragged-red fibres), NARP (neruopathy, ataxia and retinitis pigmentosa) and LHON (Leber hereditary optic neuropathy) (22).

In accordance with the present invention, a method of isolating a cytoplasmic fraction from an oocyte without impairing the capacity of the oocyte to be fertilized finds application in detecting nucleotide sequence mutations in the mitochondrial genome, enabling the skilled worker to study a mutation in the context of the functional integrity of both the oocyte and the progeny descended from the fertilized oocyte.

Thus, in a fourth aspect, the invention provides a method of detecting nucleotide sequence mutation in the mitochondrial genome of mitochondria located in an oocyte which does not impair the capacity of the oocyte to be fertilized, the method including the following steps:

a) drawing a cytoplasmic fraction which includes a sample of the mitochondria from the oocyte, into a releasing means;
b) isolating the fraction from the oocyte; and
c) analysing the nucleotide sequence of the mitochondrial genome of the mitochondria in the fraction for the presence of nucleotide sequence mutation.

In accordance with the fourth aspect of the invention, a nucleotide sequence mutation of the mitochondrial genome of the mitochondria which are in the fraction isolated from the oocyte, may be detected by use of standard techniques known in the art, and exemplified in the accompanying Examples, including techniques, or a combination of techniques, selected from the group of techniques consisting of the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing.

In accordance with the fourth aspect of the invention,

the volume of the cytoplasmic fraction drawn into the releasing means from the oocyte is typically less than 10 pL and preferably 8 pL. Preferably, the releasing means includes at least an ICSI pipette and preferably the cytoplasmic fraction is drawn approximately 100μm into the pipette.

The drawing of the cytoplasmic fraction from the oocyte into the releasing means in accordance with the fourth aspect of the invention typically forms an extrusion of cytoplasmic contents between the releasing means and the oocyte. The cytoplasmic fraction is isolated from the oocyte by gently stretching or shearing the extrusion so as to separate the extrusion. Preferably the cytoplasmic fraction is isolated by stretching the extrusion.

As mitochondrial genome mutations which cause, or are associated with, disease or dysfunction, including point mutations, are almost exclusively maternally inherited (21-22) a method of isolating a cytoplasmic fraction without impairing the capacity of the oocyte to be fertilized finds application in predicting whether the progeny descended from a fertilized oocyte contain a mutation of the mitochondrial genome which causes, or is associated, with disease or dysfunction.

Thus in a fifth aspect, the invention provides a method for predicting whether the progeny descended from a fertilized oocyte will contain a mutation in a mitochondrial genome which causes, or is associated with, a disease or dysfunction, wherein the method does not impair the capacity of the oocyte to be fertilized, and includes the following steps:

a) drawing a cytoplasmic fraction which includes a sample of mitochondria from the oocyte, into a releasing means;
b) isolating the fraction from the oocyte;
c) analysing the mitochondrial genome of the mitochondria in the fraction for the presence of the

mutation;

wherein the presence of the mutation indicates a likelihood that the progeny descended from the fertilized oocyte will contain the mutation.

In accordance with the fifth aspect of the invention, the volume of the cytoplasmic fraction drawn into the releasing means from the oocyte is typically less than 10 pL and preferably 8 pL. Preferably, the releasing means includes at least an ICSI pipette and preferably the cytoplasmic fraction is drawn approximately 100μm into the pipette.

The drawing of the cytoplasmic fraction from the oocyte into the releasing means in accordance with the fifth aspect of the invention typically forms an extrusion of cytoplasmic contents between the releasing means and the oocyte. The cytoplasmic fraction is isolated from the oocyte by gently stretching or shearing the extrusion so as to separate the extrusion. Preferably the cytoplasmic fraction is isolated by stretching the extrusion.

In accordance with the fifth aspect of the invention, the presence of the nucleotide sequence mutation, may be analysed by use of standard techniques known in the art, and exemplified in the accompanying Examples, including techniques, or a combination of techniques, selected from the group of techniques consisting of the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing.

The mere existence of a mutation in the mitochondrial genome of mitochondria in an oocyte, which is known to be associated with, or cause, disease or dysfunction, may not be sufficient to mediate the disease or dysfunction in the oocyte itself, or the progeny descended from the fertilized oocyte. Indeed, there appears to be at least an additional factor which contributes to the likelihood of, and/or the severity of the disease or dysfunction in the oocyte containing the mutation, or the progeny which are descended

from the fertilized oocyte. That is, it is likely that the actual proportion, or "threshold level", of mitochondria which contain the mutation in the mitochondrial genome in the oocyte, will contribute to the likelihood and/or severity of disease or dysfunction in the oocyte, or progeny descended from the fertilized oocyte. In particular, and in contrast with the nuclear genome, it is known that there are of the order of at least 100,000 copies of a mitochondrial genome in an oocyte (1). "Heteroplasmy" is observed when an oocyte contains more than one species of mitochondrial genome. An oocyte, and indeed, the tissues of the progeny descended from the fertilized oocyte, become "heteroplasmic" when a mutation is introduced into a cell which, before the mutation was introduced, contained a single species of mitochondrial genome. With regard to the known mutations of mitochondrial genomes which cause or are associated with a disease or dysfunction, it is generally recognised that when a particular level of heteroplasmy is surpassed, the manifestations of the disease or dysfunction are sooner or later observed (23-25).

Based on the observation that mitochondria in the mouse oocyte migrate to specific regions of the oocyte cytoplasm, in particular, the perinuclear region, at specific stages of meiosis (26), to obtain a sample which is representative of all mitochondrial genomes in the human oocyte, a method for determining the degree of heteroplasmy in a human oocyte is typically applied at specific stages of meiosis. A method of isolating cytoplasmic fractions which does not impair the capacity of the oocyte to be fertilized, is useful for determining the level of heteroplasmy in the oocyte, and for predicting the level of heteroplasmy in tissues of the progeny descended from the fertilized oocyte.

Thus, in a sixth aspect, the invention provides a method of determining the level of heteroplasmy of

mitochondrial genomes in an oocyte which does not impair the capacity of the oocyte to be fertilized, and which includes the following steps:

a) drawing a cytoplasmic fraction which includes a sample of mitochondria from the oocyte, into a releasing means;

b) isolating the fraction from the oocyte; and

c) comparing the number of mitochondrial genomes with the mutation as compared with the number of genomes without the mutation, to determine the level of heteroplasmy of genomes in the fraction.

Typically a fraction which is representative of mitochondrial genomes in the oocyte can be obtained when mitochondria are randomly distributed in the oocyte cytoplasm. Preferably a cytoplasmic fraction is drawn from the oocyte when the distribution of mitochondria in the oocyte can be expected to be random, including for example at the granular vesicle (GV) stage of the primary oocyte and/or at any stage from the metaphase II stage of meiosis of the secondary oocyte, to prior to syngamy.

In accordance with the sixth aspect of the invention, the volume of the cytoplasmic fraction drawn into the releasing means from the oocyte is typically less than 10 pL and preferably 8 pL. Preferably, the releasing means includes at least an ICSI pipette and preferablv the cytoplasmic fraction is drawn approximately 100μM into the pipette.

The drawing of the cytoplasmic fraction from the oocyte into the releasing means in accordance with the sixth aspect of the invention typically forms an extrusion of ooplasmic contents between releasing means and the oocyte. The cytoplasmic fraction is isolated from the oocyte by gently stretching or shearing the extrusion so as to separate the extrusion. Preferably the extrusion is broken by stretching the extrusion.

In accordance with the sixth aspect of the invention, the level of heteroplasmy of the mitochondria mitochondrial genome may be determined by use of standard techniques known in the art, and exemplified in the accompanying Examples, including techniques, or a combination of techniques, selected from the group of techniques consisting of the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing.

As the level of heteroplasmy and the existence of a mutation which causes, or is associated with, disease or dysfunction in progeny descended from a fertilized oocyte, contribute to the likelihood and/or severity of disease or dysfunction, the method of determining the level of heteroplasmy of mitochondrial genomes in an oocyte finds application in predicting whether the progeny descended from the fertilized oocyte are likely to suffer from a disease or dysfunction which is caused by, or associated with, the mutation, and/or the severity of the disease or dysfunction.

Thus in a seventh aspect, the invention provides a method of determining whether the progeny descended from a fertilized oocyte are likely to suffer from a disease or dysfunction caused by, or associated with, a mutation in a mitochondrial genome, wherein the method does not impair the capacity of the oocyte to be fertilized and includes the following steps:

a) drawing a cytoplasmic fraction which includes a sample of mitochondria from the oocyte, into a releasing means;
b) isolating the fraction from the oocyte;
c) analysing the mitochondrial genome of the mitochondria in the fraction for the presence of the mutation;

determining that the progeny are likely to suffer from the disease or dysfunction where the analysis of the

mitochondrial genome of mitochondria in the fraction demonstrates that the level of heteroplasmy of mitochondrial genomes with respect to the mutation in the oocyte is at least the same as the level of heteroplasmy which is known to be associated with the manifestation of the disease or dysfunction.

Typically a fraction which is representative of mitochondrial genomes in the oocyte can be obtained when mitochondria are randomly distributed in the oocyte cytoplasm. Preferably a cytoplasmic fraction is drawn from the oocyte when the distribution of mitochondria in the oocyte can be expected to be random, including for example at the granular vesicle (GV) stage of the primary oocyte and/or at any stage from the metaphase II stage of meiosis of the secondary oocyte, to prior to syngamy.

In accordance with the seventh aspect of the invention, the volume of the cytoplasmic fraction drawn into the releasing means from the oocyte is typically less than 10 pL and preferably 8 pL. Preferably, the releasing means includes at least an ICSI pipette and preferably the cytoplasmic fraction is drawn approximately 100μm into the pipette.

The drawing of the cytoplasmic fraction from the oocyte into the releasing means in accordance with the seventh aspect of the invention typically forms an extrusion of ooplasmic contents between the releasing means and the oocyte. The cytoplasmic fraction is isolated from the oocyte by gently stretching or shearing the extrusion so as to separate the extrusion. Preferably the extrusion is broken by stretching the extrusion.

In accordance with the seventh aspect of the invention, the nucleotide sequence mutation, and level of heteroplasmy of mitochondrial genomes with respect to that mutation, of the mitochondria which are in the fraction isolated from the oocyte, may be analysed by use of standard techniques known in the art, and exemplified in

the accompanying Examples, including techniques, or a combination of techniques, selected from the group of techniques consisting of the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing.

As noted above, the prior art methods for the analysis of cytoplasmic fractions from an oocyte involve the destruction of the oocyte (1-3). Although these methods reveal useful information about the oocyte which is destroyed, the value of this information is confined to an extrapolation to other oocytes which are yet to be evaluated. As oocytes from the same individual are typically heterogenous with respect to ooplasmic content, there is some question as to the extent to which the information derived from the oocyte which is destroyed can be accurately extrapolated to other oocytes. That is, using the prior art methods, the skilled worker cannot conclude that because an oocyte is found to have either no mutations in the mitochondrial genome which cause, or are associated with disease or dysfunction in the oocyte, or the progeny of the fertilized oocyte, or a low degree of heteroplasmy in relation to those mutations, that other oocytes derived from the same individual will have the same mitochondrial genotype.

Consequently, the prior art methods for the analysis of oocytes are of limited use in the field of *in vitro* fertilization, where it is anticipated that some oocytes derived from a patient may contain mutations which cause, or are associated with, disease or dysfunction in the progeny descended from the fertilized oocyte. The method of isolating a cytoplasmic fraction from an oocyte which does not impair the capacity of the oocyte to be fertilized is particularly useful for screening oocytes for the presence of mutations which cause, or are associated with, disease or dysfunction in the progeny descended from the fertilized oocyte, prior to fertilization.

Thus in an eighth aspect, the invention provides a method of screening a candidate oocyte, for the presence of mutation in the mitochondrial genome in the oocyte which causes, or is associated with, disease or dysfunction in the progeny descended from the fertilized oocyte, wherein the method does not impair the capacity of the oocyte to be fertilized, and includes the following steps:

a) drawing a cytoplasmic fraction which includes a sample of mitochondria from the oocyte, into a releasing means;
b) isolating the fraction from the oocyte; and
c) analysing the mitochondrial genome of the mitochondria in the faction for the presence of nucleotide sequence mutation.

Typically a fraction which is representative of mitochondrial genomes in the oocyte can be obtained when mitochondria are randomly distributed in the oocyte cytoplasm. Preferably a cytoplasmic fraction is drawn from the oocyte when the distribution of mitochondria in the oocyte can be expected to be random, including for example at the granular vesicle (GV) stage of the primary oocyte and/or at any stage from the metaphase II stage of meiosis of the secondary oocyte, to prior to syngamy.

In accordance with the eighth aspect of the invention, the volume of the cytoplasmic fraction drawn into the releasing means from the oocyte is typically less than 10 pL and preferably 8 pL. Preferably, the releasing means includes at least an ICSI pipette and preferably the cytoplasmic fraction is drawn approximately 100μm into the pipette.

The drawing of the cytoplasmic fraction from the oocyte into the releasing means in accordance with the eighth aspect of the invention typically forms an extrusion of ooplasmic contents between the releasing means and the oocyte. The cytoplasmic fraction is isolated from the oocyte by gently stretching or shearing the extrusion so as

to separate the extrusion. Preferably the extrusion is broken by stretching the extrusion.

In accordance with the eighth aspect of the invention, the presence of the nucleotide sequence mutation in the mitochondrial genome of the mitochondria which are in the fraction isolated from the oocyte, may be analysed by use of standard techniques known in the art, and exemplified in the accompanying Examples, including techniques, or a combination of techniques, selected from the group of techniques consisting of the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing.

Patients which present for *in vitro* fertilization treatment frequently have few oocytes available for selection for fertilization, and of the available oocytes, it is anticipated that some of these will contain a mutation in a mitochondrial genome which causes or is associated with a disease or dysfunction in progeny descended from the fertilized oocyte. The prior art methods for isolating a cytoplasmic fraction from an oocyte are particularly unsuitable for selecting oocytes for fertilization, and could potentially result in the destruction of oocytes which do not contain a mutation in a mitochondrial genome which causes or is associated with a disease or dysfunction, or which have a low level of heteroplasmy with respect to that mutation. The method of isolating a cytoplasmic fraction from an oocyte which does not impair the capacity of the oocyte to be fertilized is particularly useful for selecting oocytes for fertilization which do not contain mutations which cause or are associated with disease or dysfunction in the progeny descended from the fertilized oocyte, prior to fertilization.

Thus in a ninth aspect, the invention relates to a method of selecting an oocyte for fertilization which either:

(i) does not contain a mutation in the mitochondrial genome of mitochondria in the oocyte which causes, or is associated with, a disease or dysfunction in the progeny descended from the fertilized oocyte; or

(ii) has a level of heteroplasmy of mitochondrial genomes with respect to the mutation which is less than the level of heteroplasmy which is known to be associated with the manifestation of the disease or dysfunction;

wherein the method does not impair the capacity of the oocyte to be fertilized, and includes the following steps:

a) drawing a cytoplasmic fraction which includes a sample of mitochondria from the oocyte into a releasing means;

b) isolating the fraction from the oocyte;

c) analysing the mitochondrial genome of the mitochondria in the fraction; and

d) selecting the oocyte for fertilization, provided that at least the degree of heteroplasmy of mitochondrial genomes with respect to the mutation in the oocyte, is less than the degree of heteroplasmy which is known to be associated with the manifestation of the disease or dysfunction.

Typically a fraction which is representative of mitochondrial genomes in the oocyte can be obtained when mitochondria are randomly distributed in the oocyte cytoplasm. Preferably a cytoplasmic fraction is drawn from the oocyte when the distribution of mitochondria in the oocyte can be expected to be random, including for example at the granular vesicle (GV) stage of the primary oocyte and/or at any stage from the metaphase II stage of meiosis of the secondary oocyte, to prior to syngamy.

In accordance with the ninth aspect of the invention, the volume of the cytoplasmic fraction drawn into the

releasing means from the oocyte is typically less than 10 pL and preferably 8 pL. Preferably, the releasing means includes at least an ICSI pipette and preferably the cytoplasmic fraction is drawn approximately 100μm into the pipette.

The drawing of the cytoplasmic fraction from the oocyte into the releasing means in accordance with the ninth aspect of the invention typically forms an extrusion of ooplasmic contents between the releasing means and the oocyte. The cytoplasmic fraction is isolated from the oocyte by gently stretching or shearing the extrusion so as to separate the extrusion. Preferably the extrusion is broken by stretching the extrusion.

In accordance with the ninth aspect of the invention, the presence of the nucleotide sequence mutation in the mitochondrial genome of the mitochondria which are in the fraction isolated from the oocyte, may be analysed by use of standard techniques known in the art, and exemplified in the accompanying Examples, including techniques, or a combination of techniques, selected from the group of techniques consisting of the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing.

The oocytes which are screened or selected in accordance with the eighth or ninth aspect of the invention, respectively, may be fertilized by intra cytoplasmic sperm injection (ICSI), or by *in vitro* fertilization (4, 27) Preferably the oocytes are fertilized by ICSI.

To the extent that the ninth aspect of the invention is particularly suitable for selecting oocytes for fertilization and can be used prior to ICSI or IVF, the present inventors recognise that the method embodied in the ninth aspect of the invention is an integral part of a novel *in vitro* fertilization procedure.

Accordingly, in a tenth aspect, the invention provides a method of fertilizing an oocyte, the method including the following steps:

a) drawing a cytoplasmic fraction which includes at least some mitochondria from the oocyte into a releasing means;
b) isolating the fraction from the oocyte;
c) analysing the mitochondrial genome of the mitochondria in the fraction for the presence of a mutation which cause, or is associated with, a disease or dysfunction in progeny descended from the fertilized oocyte; and
d) provided that the degree of heteroplasmy of mitochondrial genomes with respect to the mutation in the oocyte, is less than the degree of heteroplasmy which is known to be associated with the manifestation of the disease or dysfunction, fertilizing the oocyte.

Typically a fraction which is representative of mitochondrial genomes in the oocyte can be obtained when mitochondria are randomly distributed in the oocyte cytoplasm. Preferably a cytoplasmic fraction is drawn from the oocyte when the distribution of mitochondria in the oocyte can be expected to be random, including for example at the granular vesicle (GV) stage of the primary oocyte and/or at any stage from the metaphase II stage of meiosis of the secondary oocyte, to prior to syngamy.

In accordance with the tenth aspect of the invention, the volume of the cytoplasmic fraction drawn into the releasing means from the oocyte is typically less than 10 pL and preferably 8 pL. Preferably, the releasing means includes at least an ICSI pipette and preferably the cytoplasmic fraction is drawn approximately 100μm into the pipette.

The drawing of the cytoplasmic fraction from the oocyte into the releasing means in accordance with the

tenth aspect of the invention typically forms an extrusion of ooplasmic contents between the releasing means and the oocyte. The cytoplasmic fraction is isolated from the oocyte by gently stretching or shearing the extrusion so as to separate the extrusion. Preferably the extrusion is broken by stretching the extrusion.

In accordance with the tenth aspect of the invention, the presence of the nucleotide sequence mutations of the mitochondrial genome of the mitochondria which are in the fraction isolated from the oocyte, may be analysed by use of standard techniques known in the art, and exemplified in the accompanying Examples, including techniques, or a combination of techniques, selected from the group of techniques consisting of the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing.

As the method of isolating a cytoplasmic fraction from an oocyte does not interfere significantly with the metabolism of the oocyte, the present inventors recognised that the method of isolating a cytoplasmic fraction is useful for studying the cytoplasm of other cells, for example embryonic cells, without impairing the developmental potential of those cells.

Thus, in an eleventh aspect, the invention provides a method of analysing the mitochondrial genome of mitochondria located in an embryonic cell which does not impair the developmental potential of the cell, the method including the following steps:

a) drawing a cytoplasmic fraction which includes a sample of mitochondria from the embryonic cell, into a releasing means;
b) isolating the fraction from the embryonic cell; and
c) analysing the mitochondrial genome of the mitochondria in the fraction.

In accordance with the eleventh aspect of the invention, the mitochondrial genome of the mitochondria

which are in the fraction isolated from the embryonic cell, may be analysed by use of standard techniques known in the art, and exemplified in the accompanying Examples, including techniques, or a combination of techniques, selected from the group of techniques consisting of the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing.

In accordance with the eleventh aspect of the invention, the volume of the cytoplasmic fraction drawn into the releasing means from the embryonic cell is typically less than 10 pL and preferably 8 pL. Preferably, the releasing means includes at least an ICSI pipette and preferably the cytoplasmic fraction is drawn approximately 100μm into the pipette.

The drawing of the cytoplasmic fraction from the embryonic cell into the releasing means in accordance with the eleventh aspect of the invention typically forms an extrusion of cytoplasmic contents between the distal end of the releasing means and the embryonic cell. The cytoplasmic fraction is isolated from the embryonic cell by gently stretching or shearing the extrusion so as to separate the extrusion. Preferably the cytoplasmic fraction is isolated by stretching the extrusion.

As described herein above, various deletions and point mutations of the mitochondrial genome are known to cause, or at least to be associated with, dysfunction or disease. A method of isolating a cytoplasmic fraction from an embryonic cell which does not impair the developmental potential of the cell will find application in detecting nucleotide sequence mutations in the mitochondrial genome, enabling the skilled worker to study a mutation in the context of the functional integrity of both the embryonic cell and the progeny descended from the embryonic cell.

Thus in a twelfth aspect, the invention provides a method of detecting nucleotide sequence mutation in the

mitochondrial genome of mitochondria located in an embryonic cell which does not impair the developmental potential of the cell, the method including the following steps:

a) drawing a cytoplasmic fraction which includes a sample of the mitochondria from the embryonic cell, into a releasing means;

b) isolating the fraction from the embryonic cell; and

c) analysing the nucleotide sequence of the mitochondrial genome of the mitochondria in the fraction for the presence of nucleotide sequence mutation.

In accordance with the twelfth aspect of the invention, a nucleotide sequence mutation of the mitochondrial genome of the mitochondria which are in the fraction isolated from the embryonic cell, may be detected by use of standard techniques known in the art, and exemplified in the accompanying Examples, including techniques, or a combination of techniques, selected from the group of techniques consisting of the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing.

In accordance with the twelfth aspect of the invention, the volume of the cytoplasmic fraction drawn into the releasing means from the embryonic cell is typically less than 10 pL and preferably 8 pL. Preferably, the releasing means includes at least an ICSI pipette and preferably the cytoplasmic fraction is drawn approximately 100μm into the pipette.

The drawing of the cytoplasmic fraction from the embryonic cell into the releasing means in accordance with the twelfth aspect of the invention typically forms an extrusion of cytoplasmic contents between the releasing means and the embryonic cell. The cytoplasmic fraction is isolated from the embryonic cell by gently stretching or shearing the extrusion so as to separate the extrusion.

Preferably the cytoplasmic fraction is isolated by stretching the extrusion.

The present inventors recognised that a method of isolating a cytoplasmic fraction without impairing the developmental potential of an embryonic cell finds application in predicting whether the progeny descended from the embryonic cell will contain a mutation of the mitochondrial genome which causes, or is associated, with disease or dysfunction.

Thus, in a thirteenth aspect, the invention provides a method of predicting whether the progeny descended from an embryonic cell will contain a mutation in a mitochondrial genome which causes, or is associated with, a disease or dysfunction, wherein the method does not impair the developmental potential of the cell, and includes the following steps:

a) drawing a cytoplasmic fraction which includes a sample of mitochondria from the embryonic cell, into a releasing means;
b) isolating the fraction from the embryonic cell;
c) analysing the mitochondrial genome of the mitochondria in the fraction for the presence of the mutation;

wherein the presence of the mutation indicates a likelihood that the progeny descended from the embryonic cell will contain the mutation.

In accordance with the thirteenth aspect of the invention, the volume of the cytoplasmic fraction drawn into the releasing means from the embryonic cell is typically less than 10 pL and preferably 8 pL. Preferably, the releasing means includes at least an ICSI pipette and preferably the cytoplasmic fraction is drawn approximately 100μm into the pipette.

The drawing of the cytoplasmic fraction from the embryonic cell into the releasing means in accordance with the thirteenth aspect of the invention typically forms an

extrusion of cytoplasmic contents between the releasing means and the embryonic cell. The cytoplasmic fraction is isolated from the embryonic cell by gently stretching or shearing the extrusion so as to separate the extrusion. Preferably the cytoplasmic fraction is isolated by stretching the extrusion.

In accordance with the thirteenth aspect of the invention, the presence of the nucleotide sequence mutation, may be analysed by use of standard techniques known in the art, and exemplified in the accompanying Examples, including techniques, or a combination of techniques, selected from the group of techniques consisting of the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing.

As discussed herein above, it is likely that a particular level of heteroplasmy must be surpassed before the manifestations of disease or dysfunction are observed, which are caused by, or associated with, a mitochondrial genome mutation.

Thus, in a fourteenth aspect, the invention provides a method of determining the level of heteroplasmy of mitochondrial genomes in an embryonic cell which does not impair the developmental potential of the cell, and which includes the following steps

a) drawing a cytoplasmic fraction which includes a sample of mitochondria from the embryonic cell, into a releasing means;
b) isolating the fraction from the embryonic cell; and
c) comparing the number of mitochondrial genomes with the mutation as compared with the number of genomes without the mutation, to determine the level of heteroplasmy of genomes in the fraction.

In accordance with the fourteenth aspect of the invention, the volume of the cytoplasmic fraction drawn into the releasing means from the embryonic cell is

typically less than 10 pL and preferably 8 pL. Preferably, the releasing means includes at least an ICSI pipette and preferably the cytoplasmic fraction is drawn approximately 100μM into the pipette.

The drawing of the cytoplasmic fraction from the embryonic cell into the releasing means in accordance with the fourteenth aspect of the invention typically forms an extrusion of cytoplasmic contents between releasing means and the embryonic cell. The cytoplasmic fraction is isolated from the embryonic cell by gently stretching or shearing the extrusion so as to separate the extrusion. Preferably the extrusion is broken by stretching the extrusion.

In accordance with the fourteenth aspect of the invention, the level of heteroplasmy of the mitochondrial genome may be determined by use of standard techniques known in the art, and exemplified in the accompanying Examples, including techniques, or a combination of techniques, selected from the group of techniques consisting of the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing.

In a fifteenth aspect, the invention provides a method of determining whether the progeny descended from an embryonic cell are likely to suffer from a disease or dysfunction caused by, or associated with, a mutation in a mitochondrial genome, wherein the method does not impair the developmental potential of the cell, and includes the following steps:

a) drawing a cytoplasmic fraction which includes a sample of mitochondria from the embryonic cell, into a releasing means;
b) isolating the fraction from the embryonic cell;
c) analysing the mitochondrial genome of the mitochondria in the fraction for the presence of the mutation;

determining that the progeny are likely to suffer from the disease or dysfunction where the analysis of the mitochondrial genome of mitochondria in the fraction demonstrates that the level of heteroplasmy of mitochondrial genomes with respect to the mutation in the embryonic cell is at least the same as the level of heteroplasmy which is known to be associated with the manifestation of the disease or dysfunction.

In accordance with the fifteenth aspect of the invention, the volume of the cytoplasmic fraction drawn into the releasing means from the embryonic cell is typically less than 10 pL and preferably 8 pL. Preferably, the releasing means includes at least an ICSI pipette and preferably the cytoplasmic fraction is drawn approximately 100μm into the pipette.

The drawing of the cytoplasmic fraction from the embryonic cell into the releasing means in accordance with the fifteenth aspect of the invention typically forms an extrusion of cytoplasmic contents between the releasing means and the embryonic cell. The cytoplasmic fraction is isolated from the embryonic cell by gently stretching or shearing the extrusion so as to separate the extrusion. Preferably the extrusion is broken by stretching the extrusion.

In accordance with the fifteenth aspect of the invention, the nucleotide sequence mutation, and level of heteroplasmy of mitochondrial genomes with respect to that mutation, of the mitochondria which are in the fraction isolated from the embryonic cell, may be analysed by use of standard techniques known in the art, and exemplified in the accompanying Examples, including techniques, or a combination of techniques, selected from the group of techniques consisting of the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing.

Prior to commencing the embryo transfer procedure it is important to select (i) an embryo which does not contain a mitochondrial mutation which causes, or is associated with, disease or dysfunction, and/or (ii) an embryo which has a low level of heteroplasmy with respect to that mutation. The method of isolating a cytoplasmic fraction from an embryonic cell which does not impair the developmental potential of the cell is particularly useful for selecting an embryo for embryo transfer which does not contain mutations which cause or are associated with disease or dysfunction in the progeny descended from the embryonic cell, prior to embryo transfer.

Thus, in a sixteenth aspect, the invention provides a method of selecting an embryo for embryo transfer, which either:

(i) does not contain a mutation in the mitochondrial genome of mitochondria in an embryonic cell derived from the embryo, which mutation causes, or is associated with, a disease or dysfunction in the progeny descended from the embryonic cell or embryo; or

(ii) has a level of heteroplasmy of mitochondrial genomes with respect to the mutation which is less than the level of heteroplasmy which is known to be associated with the manifestation of the disease or dysfunction;

wherein the method does not impair the developmental potential of the embryo, and includes the following steps:

a) drawing a cytoplasmic fraction which includes a sample of mitochondria from an embryonic cell derived from the embryo into a releasing means;

b) isolating the fraction from the embryonic cell;

c) analysing the mitochondrial genome of the mitochondria in the fraction; and

selecting the embryo for embryo transfer, provided that at least the degree of heteroplasmy of mitochondrial genomes with respect to the mutation in the embryonic cell, is less than the degree of heteroplasmy which is known to be associated with the manifestation of the disease or dysfunction.

In accordance with the sixteenth aspect of the invention, the volume of the cytoplasmic fraction drawn into the releasing means from the embryonic cell is typically less than 10 pL and preferably 8 pL. Preferably, the releasing means includes at least an ICSI pipette and preferably the cytoplasmic fraction is drawn approximately 100μm into the pipette.

The drawing of the cytoplasmic fraction from the embryonic cell into the releasing means in accordance with the sixteenth aspect of the invention typically forms an extrusion of cytoplasmic contents between the releasing means and the embryonic cell. The cytoplasmic fraction is isolated from the embryonic cell by gently stretching or shearing the extrusion so as to separate the extrusion. Preferably the extrusion is broken by stretching the extrusion.

In accordance with the sixteenth aspect of the invention, the nucleotide sequence mutation, and level of heteroplasmy of mitochondrial genomes with respect to that mutation, of the mitochondria which are in the fraction isolated from the embryonic cell, may be analysed by use of standard techniques known in the art, and exemplified in the accompanying Examples, including techniques, or a combination of techniques, selected from the group of techniques consisting of the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing.

In an seventeenth aspect, the invention relates to the use of a releasing means for isolating a cytoplasmic fraction from an oocyte, or from an embryonic cell.

Typically, the releasing means includes an injection pipette. Preferably, the injection pipette is an ISCI pipette.

In a nineteenth aspect, the invention relates to a kit for use in the method of isolating a cytoplasmic fraction from an oocyte, or from an embryonic cell, which includes at least one oligonucleotide probe specific for a nucleotide sequence mutation in the mitochondrial genome of mitochondria in an oocyte or embryonic cell, which mutation causes, or which is associated with, a disease or dysfunction in the progeny descended from the fertilized oocyte or embryonic cell. In one embodiment, the at least one oligonucleotide probe is specific for any one of the nucleotide sequence mutations in the mitochondrial genome shown in Table 1.

Table 1

Position	RNA	Gene	Disease	
721	r	12s	ADPD	Alzheimers Parkinsons
1555	r	12s	DEAF	*weak*
1642	t	Val	MELAS	
3010	r	16s	longevity	
3196	r	16s	ADPD	
3243	t	Leu(UUR)	MELAS	Mitochondrial encephalopathy, lactic acidosis, stroke-like episodes
3250	t	Leu(UUR)	MM	Mitochondrial myopathy
3251	t	Leu(UUR)	MM	Mitochondrial myopathy
3254	t	Leu(UUR)	diabetes	
3260	t	Leu(UUR)	HCM	Hypertrophic cardiomyopathy
3271	t	Leu(UUR)	MELAS	*later onset than 3242*
3302	t	Leu(UUR)	MM	
3303	t	Leu(UUR)	HCM	

3394	m	ND1	LHON		
3397	m	ND1	ADPD		
3460	m	ND1	LHON		*severe*
4160	m	ND1	LHON+		
4216	m	ND1	LHON		
4269	t	Ile	FICP	Fatal infantile cardiomyopathy plus	
4317	t	Ile	FICP		
4336	t	Gln	ADPD		*weak*
4917	m	ND2	LHON		
5178	m	ND2	longevity		
5244	m	ND2	LHON		
5521	t	Trp		Myopathy, late-onset	
6480	m	COI			
7444	m	COI	LHON		
7445	m	COI	PPK	Palmoplantar keratoderma & hearing loss	
7472	t	Ser		Myoclonus epilepsy, w/o RRF	
7497	t	Ser	MERRF	Myoclonus epilepsy etc	
7512	t	Ser		Myoclonus epilepsy, w/o RRF	
8344	t	Lys	MERRF	Myoclonic epilepsy, ragged red fibres; lipomatosis	
8356	t	Lys	MERRF		
8414	m	ATP6	longevity		
8851		ATP6		Bilateral striatal necrosis	
8993	m	ATP6	NARP/Leighs	Neurogenic weakness, ataxia, retinitis	
9176	m	ATP6		Bilateral striatal necrosis	
9438	m	COI	LHON		
9804	m	COI	LHON		
9997	t	Gly	HCM		

10004	t	Gly		Sudden childhood death?
11778	m	ND4	LHON	*severe*
13708	m	ND5	LHON	
13730	m	ND5	LHON	
14459	m	ND6	LHON	
14484	m	ND6	LHON	*severe*
14709	t	Glu		Varied: infantile myopathy to NIDDM
15257	m	cyt b	LHON	
15762	m	cyt b		Myopathy, late-onset
15812	m	cyt b	LHON	
15923	t	Thr	LIMM	Lethal infantile mitochondrial myopathy
15990	t	Pro	MM	
16189	D		NIDDM	Non-insulin dependent diabetes

N= any nucleotide

R= either puRine (A,G)

Y= either pYrimidine (U,C)

In a twentieth aspect, the invention relates to oligonucleotides including the following sequences:

ATP6F: TCACCACCCAACAATGAC

ATP6R: TAAGGCGACAGCGATTTC.

DEFINITIONS:

In the specification and claims, "oocyte" means a female germ line cell including primary oocytes and secondary oocytes, and includes human oocytes. Primary oocytes include germ cells at the GV (granular vesicle) stage of meiosis. Secondary oocytes include germ cells at the metaphase II stage of meiosis.

In the specification and claims, "progeny descended from a fertilized oocyte" means the individual which is generated from the fertilization of the female germ cell with the male germ cell. "Individual" means the multi-cellular organism from the earliest stage of embryonic life (for example, the 2 cell stage) to adult life.

In the specification and claims, "does not impair the capacity of the oocyte to be fertilized" means that the oocyte which has had a fraction of cytoplasm isolated, may be fertilized, or in other words, may undergo any one or more of the biochemical or cellular events which are associated with any one or more of the stages of fertilization, from the activation of the oocyte by entry of sperm, to the generation of a zygote and the formation of cleavage products of the zygote. Typically after the fraction of the cytoplasm is isolated, the oocyte may be fertilized *in vitro* by standard techniques, including for example ICSI and IVF.

In the specification and claims, "embryonic cell" includes a post-syngamous fusion product of the female and male germ cells, a zygote, and cleavage products of a zygote at any stage of development from the 2 cell stage to the stage of implantation.

In the specification and claims, "progeny descended from an embryonic cell" or "progeny descended from an embryo" means the individual which is generated from the post syngamous fusion product of the female and male germ cells. "Individual" means the multi-cellular organism from the earliest stage of embryonic life (for example, the 2 cell stage) to adult life.

In the specification and claims "does not impair the developmental potential of the cell" or "does not impair the developmental potential of the embryo" means that the embryonic cell which has had a fraction of cytoplasm isolated, and the embryo from which the embryonic cell may be derived, may undergo any one or more of the biochemical

or cellular events which are associated with cell differentiation and/or maturation.

BRIEF DESCRIPTION OF FIGURES

Figure 1 shows a 400 bp fragment amplified from the D-loop region of the mitochondrial genome derived from cytoplasmic biopsy samples of oocytes A1 to A7, using oligonucleotide primers L29 and H04.

BEST METHOD FOR CARRYING OUT THE INVENTION

MATERIALS AND METHODS

Source of oocytes

Human oocytes A1 to A5 were donated for research. These oocytes were either granular vesicle (GV) cells or were at the MI stage of meiosis, and were 6 hours post-retrieval.

Human oocytes B1 to B8 were donated for research. These oocytes were at the MII stage of meiosis and were 24 hours post-retrieval.

Human oocyte C1 was designated at the patients request to be part of the study. This oocyte was at the MII stage of meiosis and was 24 hours post-retrieval.

Isolation of oocyte cytoplasmic fractions

A small amount of ooplasmic material was biopsied from human oocytes using an ICSI pipette (Sydney IVF, Sydney). Briefly, the pipette is a glass capillary drawn out to have an end diameter of approximately 7μm with a bevelled tip.

The biopsy technique was performed as follows: ooplasm was drawn into the pipette to a distance of approximately 100μm (approximately 8pl). The pipette was then withdrawn from the oocyte, forming a thin ooplasmic bridge, which was then broken by stretching. Each ooplasmic biopsy was expelled directly into a PCR tube

containing 20µl of PCR buffer, Proteinase K and 20 mM DTT. Tubes were incubated either at 37°C overnight, or at 50°C for 30 minutes and then frozen. Both protocols were followed with heat inactivation of Proteinase K at 95°C for 10 minutes.

Analysis of mitochondrial genome in oocyte cytoplasmic fractions

The mitochondrial genome in the oocyte cytoplasmic fractions was analysed by the polymerase chain reaction (PCR).

(i) D-loop fragment

Reactions of 20µl were established; 10 µl of reaction mixture was added to 10µl of ooplasmic biopsy preparation. The 10µl reaction mixture contained 2.5 pmol of each primer, 200µM of each dNTP, PCR buffer, milli-Q water and 0.5 units of Taq. All reactions were carried out in capped 0.2ml tube strips. PCR cycling was performed in an FTS Thermal Sequencer (Corbett Research, Sydney, NSW) under the following conditions: initial denaturation at 93°C for 5 minutes, followed by 24 to 40 cycles of 93°C denaturation for 45 seconds, 60°C annealing for 1 minute and 72°C extension for 1 minute; ending with a polishing step of 72°C for 7 minutes, cooling to 15°C and holding at 4°C. Complete reaction mixtures lacking template DNA were included in all PCR reactions as negative controls.

Primers L29 (5'- GGTCTATCACCCTATTAACCAC-3') and H04 (5'-CTGTTAAAAGTGCATACCGCCA-3'), specific for a 400 bp sequence in the mitochondrial D-loop region were used.

(ii) Common deletion fragment

An identical protocol to that described above was used, but with the following conditions: initial denaturation at 95°C for 3 minutes followed by 20 to 35 cycles of 92°C denaturation for 1 minute, 60°C annealing for 10 seconds and 68°C extension for 45 seconds; ending with a

polishing step of 75°C for 7 minutes, cooling to 15°C and holding at 4°C. From cycle 11, 15 seconds was added to the extension time every cycle. Complete reaction mixtures lacking template DNA were included in all PCR reactions as negative controls.

Primers L820 (5'-TTCATGCCCATCGTCCTAGA-3') and H1363 (5'-GGGGAAGGGAGGTTGACCTG-3'), specific for the 4977 bp common deletion region, require the use of the modified enzyme, Expand™ High Fidelity, due to the large fragment size being amplified and the specialised 'long' PCR program.

The amplified DNA fragments from the D-loop region or the common deletion region were analysed by polyacrylamide gel electrophoresis on a 5% 37:1 acrylamide: bisacrylamide gel.

Insemination of biopsied oocytes

The B1 to B8 oocytes and the C1 oocyte were fertilized by either ICSI or IVF according to standard protocols (28,29). The insemination was performed immediately after cytoplasmic biopsy of the oocyte.

RESULTS

Oocyte fate after cytoplasmic biopsy

Oocytes A1 to A5 were not visibly affected by the cytoplasmic biopsy procedure and showed no signs of degeneration at 48 hours after biopsy. Oocytes B1 to B3 and B5 to B8 showed no signs of degeneration at 48 hours after biopsy. Oocyte C1 showed no signs of degeneration at 48 hours after biopsy. A single oocyte, B4, degenerated at 17 hours after biopsy (Table 2).

Table 2

Oocyte	Cytoplasmic Biopsy	17 hour post biopsy	48 hour post biopsy
A1	YES		No degeneration
A2	YES		No degeneration
A3	YES		No degeneration

A4	YES		No degeneration
A5	YES		No degeneration
B1	YES		No degeneration
B2	YES		No degeneration
B3	YES		No degeneration
B4	YES	Degenerated	
B5	YES		No degeneration
B6	YES		No degeneration
B7	YES		No degeneration
B8	YES		No degeneration
C1	YES		No degeneration

PCR amplification of mitochondrial DNA from cytoplasmic biopsy

Consideration was given to the amplification of mitochondrial DNA from a cytoplasmic biopsy taken from an oocyte. It was necessary to calculate the approximate number of mitochondria that would probably be obtained in such a biopsy to determine whether there would be sufficient template DNA for the PCR.

The cytoplasmic biopsy was obtained by using an ICSI pipette as described above. It was estimated that the volume of this cytoplasmic biopsy would be approximately 8pl. As the volume of an oocyte is approximately 500pl, the cytoplasmic biopsy removed is estimated to comprise approximately 2% of the cytoplasm. In a conservative estimate, there are approximately 100,000 mitochondrial genomes per oocyte (1). In accordance with this estimate, the biopsy would remove approximately 1000 mitochondria. This amount of mitochondria is within the amplification capabilities of the polymerase chain reaction.

PCR amplification from the D-loop region

A 400 bp D-loop fragment was amplified from the cytoplasmic biopsy of oocytes A1 to A5 with 35 cycles of amplification (Figure 1).

PCR amplification from the common deletion region

A 5.5kb fragment was amplified from the cytoplasmic biopsy of oocytes B1 to B3 and B5 to B8 (data not shown).

Insemination of biopsied oocytes

The results of the insemination of the biopsied oocytes, B1 to B8 and C1 are shown in Table 3.

Table 3

Oocyte	IVF/ICSI	17 hour post biopsy	48 hour post biopsy
B1	ICSI	2PN	2 cell
B2	ICSI	2PN	No division
B3	ICSI	3PN	2 cell
B4	ICSI	Degenerated	
B5	IVF	Not fertilized	
B6	IVF	Not fertilized	
B7	IVF	Not fertilized	
B8	IVF	Not fertilized	
C1	ICSI	2PN	2 cell

PN= pronuclei observed

All oocytes that were subject to the ICSI protocol, (except B4) were fertilized. Two pro-nuclei were observed in B1, B3 and C1, and 3 pro-nuclei were observed in B3 at 17 hours after fertilization. The oocytes B1, B3 and C1 divided and progressed to the 2 cell stage at 48 hours post fertilization. No cell division was observed in B2 at 48 hours.

Although the B5 to B8 oocytes had not degenerated at 48 hours post biopsy, none of these oocytes were fertilized by insemination via the IVF protocol.

DISCUSSION

Degeneration was observed in only one of the 14 oocytes which were subjected to the cytoplasmic biopsy technique. As some oocytes tend to degenerate at approximately 24 hours post retrieval (32,33), it is possible that the B4 oocyte degenerated independently of the cytoplasmic biopsy technique. It should be noted that the oocytes A1 to A5 were matured in a medium formulated

specifically for insemination, rather than oocyte maturation.

The results show that the cytoplasmic biopsy technique can be generally applied up to 24 hours post retrieval of the oocyte. The biopsy technique is therefore able to be used together with known fertilization techniques, including for example, ICSI and IVF, which are generally used approximately 4 to 6 hours post retrieval of the oocyte. Although in the present study, insemination was performed immediately after biopsy, it is expected that the biopsy may be performed after insemination.

We have found that the percentage of oocytes that are morphologically intact after the ICSI procedure (i.e the injection of sperm without removal of cytoplasm) is 95.2% (based on 2649 oocytes injected) (unpublished results).

By way of comparison, the standard for other types of micro-manipulation was difficult to establish, with the rate of degeneration after polar body and underlying cytoplasm removal for nuclear transplantation in other species not being documented (30, 31). A survival rate of 30.8% (67/217) quoted for foreign mitochondrial injection and a 6% lysis rate has been reported with ooplasmic injection in human oocytes.

Although removal of the cytoplasm is more invasive than ICSI alone, the overall oocyte survival rate in the present study is surprisingly higher than our above discussed survival rates after standard ICSI. Thus the cytoplasmic biopsy technique can be considered potentially acceptable clinically.

The amplification of fragments from the D loop region and the common deletion region of the mitochondrial genome from mitochondria in the cytoplasmic biopsy demonstrates that there is a sufficient source of template DNA in the biopsy sample for analysis by PCR, and that fragments of the order of from less than 0.5 kb to greater than 5 kb can be amplified from the sample. The amplification of

fragments from 2 independent loci of the mitochondrial genome suggests that mutations at other loci of the mitochondrial genome, in particular the mutations described in Table 1, can be amplified from the cytoplasmic biopsy sample using PCR and specific oligonucleotides.

The fertilization rate and subsequent cleavage rate was encouraging considering the oocytes donated in these experimental cohorts were greater than 24 hours old at the time of cytoplasmic biopsy and attempted fertilization. Previous studies have indicated that culture for a period exceeding 20 hours before insemination can compromise oocyte fertilization and development (32,33). In addition, oocytes matured *in vitro* lack the capacity of oocytes matured *in vivo* to maintain a high rate of cleavage (34). For this reason, it was not surprising that the B5 to B8 oocytes were not fertilized subsequent to insemination via the IVF protocol.

REFERENCES:

1. Chen, X., Prosser, R., Simonetti, S., Sadlock, J., Jagiello, G., and Schon, E. A. Rearranged mitochondrial genomes are present in human oocytes. *Am.J.Hum.Genet.* 1995; **57**:239-247.

2. Keefe, D. L., Niven-Fairchild, T., Powell, S., and Buradagunta, S. Mitochondrial deoxyribonucleic acid deletions in oocytes and reproductive aging in women. *Fertil.Steril.* 1995; **64**:577-583.

3. Müller-Hocker, J., Schafer, S., Weis, S., Munscher, C., and Strowitzki, T. Morphological-cytochemical and molecular genetic analyses of mitochondria in isolated human oocytes in the reproductive age. *Mol.Hum.Reprod.* 1996; **2**:951-958.

4. Palermo, G., Joris, H., Devroey, P. And Van Steirteghem, A. C. Pregnancies after intracytoplasmic injection of a single spermatozoon into an oocyte. The Lancet. 1992; **340:** 17.

5. Schon, E. A. Mitochondria In: DiMauro, S. and Wallace, D. C. (editors) *Mitochondrial DNA in Human Pathology* . New York: Raven Press; 1993; pp 1-7.

6. Zeviani, M., Amati, P., Comi, G., Fratta, G., Mariotti, C., and Tiranti, V. Searching for genes affecting the structural integrity of the mitochondrial genome. *Biochem. Biophys. Acta Mol. Basis Dis.*; 1995; **1271:** 153-158.

7. Klopstock, T., Bischof, F. Gerok, K., Deuschl, G., Seibel, P., Ketelsen, U.-P and Reichmann, J. 3.1-kb deletion of mitochondrial DNA in a patient with Kearns-Sayre syndrome. *Acta Neuropathol.* (Berl.) 1995; **90:** 126-129.

8. Vazquez-Acevedo, M., Coria, R., Gonzalez-Astiazaran, A., Medina-Crespo, V., Ridaura-Sanz, C. And Gonzalez-Halphen, D. Characterisation of a 5025 base pair mitochondrial DNA dleletion in Kearns-Sayre syndrome. *Biochim. Biophys. Acta Mol. Basis Dis.* 1995; **1271:** 363-368.

9. Kleinle, S., Wiesmann, U., Superti-Furga, A., Karhenbuhl, S., Boltshauser, E., Reichen, J., and Liechti-Gallati, S. Detection and characterization of mitochondrial DNA rearrangements in Pearson and Kearns-Sayre syndromes by long PCR. *Human Genetics*. 1997; **100:**643-650.

10. Corral-Debrinski, M., Shoffner, J. M., Lott, M. T., and Wallace, D. C. Association of mitochondrial DNA damage

with aging and coronary atherosclerotic heart disease. *Mutat.Res.* 1992; **275**:169-180.

11. Hayakawa, M., Hattori, K., Sugiyama, S., and Ozawa, T. Age-associated oxygen damage and mutations in mitochondrial DNA in human hearts. *Biochem.Biophys.Res.Commun.* 1992; **189**:979-985.

12. Munscher, C., Müller-Hocker, J., and Kadenbach, B. Human aging is associated with various point mutations in tRNA genes of mitochondrial DNA. *Biol.Chem.Hoppe-Seyler* 1993; **374**:1099-1104.

13. Melov, S., Shoffner, J. M., Kaufman, A., and Wallace, D. C. Marked increase in the number and variety of mitochondrial DNA rearrangements in aging human skeletal muscle. *Nucleic Acids Res.* 1995; **23**:4122-4126.

14. Cortopassi, G. A. and Arnheim, N. Detection of a specific mitochondrial DNA deletion in tissues of older humans. *Nucleic Acids Res.* 1990; **18**:6927-6933.

15. Sugiyama, S., Hattori, K., Hayakawa, M., and Ozawa, T. Quantitative analysis of age-associated accumulation of mitochondrial DNA with deletion in human hearts. *Biochem.Biophys.Res.Commun.* 1991; **180**:894-899.

16. Soong, N. W., Hinton, D. R., Cortopassi, G. A., and Arnheim, N. Mosaicism for a specific somatic mitochondrial DNA mutation in adult human brain. *Nature Genet.* 1992; **2**:318-323.

17. Corral-Debrinski, M., Horton, T., and Lott, M. T. Mitochondrial DNA deletion in human brain: regional variability and increase with advanced age. *Nature Genet.* 1992; **2**:324-329.

18. Kato, T. and Takahashi, Y. Deletion of leukocyte mitochondrial DNA in bipolar disorder. *J.Affect.Disord.* 1996; **37**:67-73.

19. Barreau, E., Brossas, J. Y., Courtois, Y., and Tréton, J. A. Accumulation of mitochondrial DNA deletions in human retina during aging. *Invest.Ophthalmol.Vis.Sci.* 1996; **37**:384-391.

20. Kitagawa, T., Suganuma, N., Nawa, A., Kikkawa, F., Tanaka, M., Ozawa, T., and Tomoda, Y. Rapid accumulation of deleted mitochondrial deoxyribonucleic acid in postmenopausal ovaries. *Biol.Reprod.* 1993; **49**:730-736.

21. Pallotti, F., Chen, X., Bonilla, E., and Schon, E. A. Evidence that specific mtDNA point mutations may not accumulate in skeletal muscle during normal human aging. *Am.J.Hum.Genet.* 1996; **59**:591-602.

22. Wallace, D. C., Lott, M. T., Brown, M. D., Huoponen, K., and Torroni, A. Human gene mapping 1995: a compendium In: Cuticchia, A. J. (editors) Baltimore: Hopkins University Press; 1995; pp 910-954.

23. Freckmann, M. L., Thorburn, D. R., Kirby, D. M., Kamath, K. R., Hammond, J., Dennett, X., and Christodoulou, J. Mitochondrial electron transport chain defect presenting as hypoglycemia. *J.Pediat.* 1997; **130**:431-436.

24. Shoffner, J. M. and Wallace, D. C. Oxidative phosphorylation diseases. *Adv.Hum.Genet.* 1990; **19**:267-330.

25. Edris, W., Burgett, B., Stine, O. C., and Filburn, C. R. Detection and quantitation by competitive PCR of an

age associated increase in a 4.8-kb deletion in rat mitochondrial DNA. *Mutat.Res.* 1994; **316**:69-78.

26. Van Blerkom, J. and Runner, M. N. Mitochondrial reorganization during resumption of arrested meiosis in the mouse oocyte. *Am.J.Anat.* 1984; **171**:335-355.

27. Steptoe, P. and Edwards, R. Pregnancy in an infertile patient after transfer of an embryo fertilized *in vitro*. *Brit. Med. J. of Clin. Res.* 1983; **286:** 1351-1352.

28. "Intracytoplasmic Sperm Injection, The Revolution in Male Infertility" Edited by Flaherty, S. P. and Matthews, C. D. in *Reproduction, Fertility and Development*, Vol. 7, No. 2, 1995, CSIRO Australia.

29. Brinsden, P.R. and Rainsbury P. A., editors. A textbook of in vitro fertilization and assisted reproduction. 1ed. Lancashire: The Parthenon Publishing Group Ltd. 1992

30. Meng, L., Ely, J. J., Souffer, R. L. and Wolf, D. P. Rhesus monkeys produced by nuclear transfer. *Biol. Reprod.* 1997; **57:**454-459.

31. Lavoir, M. C., Kelk, D., Rumph, N., Barnes, F., Betteridge, K. J., and King, W. A. Transcription and translation in bovine nuclear transfer embryos. *Biol. Reprod.* 1997; **57:**204-213.

32. Trounson, A., O., Mohr, L. R., Wood, C., and Leeton, J. F. Effect of delayed insemination on in vitro fertilization, culture and transfer of human embryos. *J. Reprod. Fertil.* 1982; **64:** 294

33. Plachot, M., de Grouch, J., Junca, A. M., Mandelbaum, J., Salat-Baroux, J., and Cohen, J. Chromosomal analysis of human oocytes and embryos: Does delayed

fertilization increase chromosome imbalance? *Hum. Reprod.*; **32:**125-127.

34. Trounson, A., Anderiesz, C., Carson, R., Jones, G., McShane, P., Lolatgis, N., and Wood, C. Oocyte maturation and growth of human embryos to blastocysts in vitro. (Abstract) *J. Ass Reprod. Genet.* 1997; **14:** 472-473.

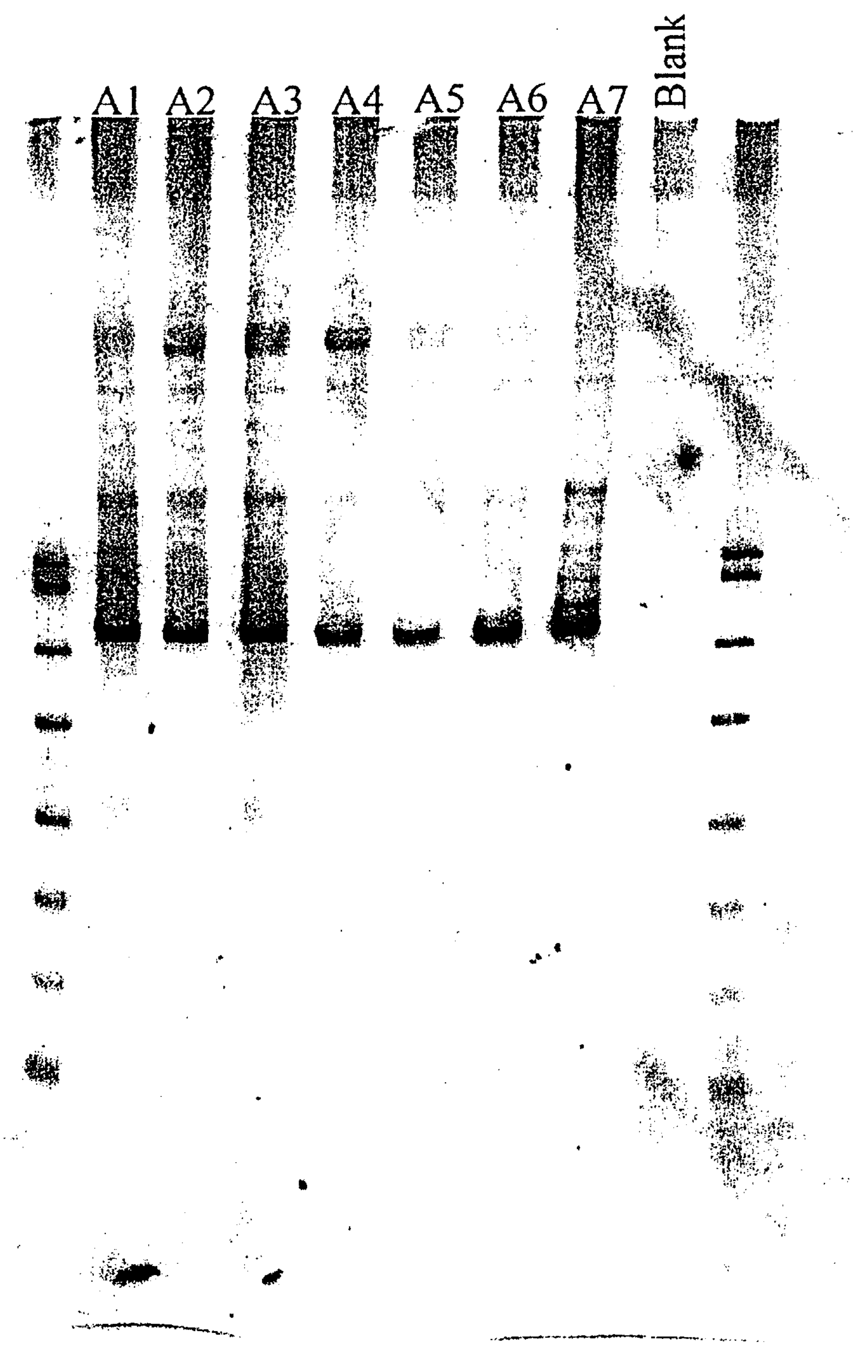

FIGURE 1